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Antifungal activity of geraniol and citronellol against food-relevant dematiaceous fungi *Cladosporium* spp.

Atividade antifúngica de geraniol e citronelol frente a fungos dematiáceos de importância para os alimentos do gênero *Cladosporium* spp.

Aldeir Sabino dos SANTOS[1], Gezaíldo Santos SILVA[1], Kaltz Victor Souza SILVA[1], Maria Islaine de Oliveira LIMA[1], Juliana Moura Mendes ARRUA[3], Edeltrudes de Oliveira LIMA[2], Fillipe de Oliveira PEREIRA[1]*

*Correspondence to: [1]Centro de Educação e Saúde, Universidade Federal de Campina Grande, Sítio Olho D'Água da Bica, s/n, Zona Rural, Cuité, PB, Brasil, CEP: 58175-000. Tel: 55 83 3372 1900. Email: fillipeopereira@ufcg.edu.br

[2]Laboratório de Micologia, Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba, Campus Universitário Castelo Branco, João Pessoa, PB, Brasil

[3]Centro Multidisciplinario de Investigaciones Tecnólogicas, Universidad Nacional de Asunción, San Lorenzo, Paraguay

ABSTRACT

Cladosporium spp. is a group of dematiaceous food-relevant fungi which are well dispersed in the environment causing food spoilage and poisoning. Considering the importance of fungal contamination, natural drugs to control their growth have become important. Thus, the aim of this study was to evaluate the inhibitory effects of two monoterpenoids, (geraniol and citronellol), against strains of *Cladosporium carrioni, C. cladosporioides,* and *C. oxysporum*. Methods: The Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC) of the drugs were determined by microdilution. The effects of test drugs on mycelial dry weight, conidia germination, and conidiogenesis of *Cladosporium* spp. were also investigated using a hemacytometer. Respective MIC and MFC values of citronellol varied from 256 to 512 µg/mL, and from 256 to 2048 µg/mL. The MIC and MFC of geraniol varied similarly to citronellol. Conidia germination, mycelial dry weight, and conidiogenesis of *Cladosporium* spp. were reduced by the test-drugs at 1/2MIC, MIC and 2xMIC ($p<0.05$). These measurable cell events are essential for fungal infection and development in foods. The action of citronellol and geraniol against *Cladosporium* spp. suggest that the drugs may serve as effective agents for controlling fungal contamination and growth in foods.

Keywords. monoterpenes, contamination, natural products, food safety, *Cladosporium*.

RESUMO

Cladosporium spp. é um grupo de fungos dematiáceos relevantes para os alimentos, que podem ser dispersos pelo ambiente e causar deterioração e intoxicação alimentar. Considerando a importância da contaminação fúngica, os produtos naturais usados para controlar seu crescimento são importantes. Neste contexto, o objetivo deste estudo foi avaliar os efeitos inibitórios de dois monoterpenoides, geraniol e citronelol, contra cepas de *Cladosporium carrioni, C. cladosporioides* e *C. oxysporum*. A Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima (CFM) das drogas foram determinadas por microdiluição. Os efeitos das drogas-teste sobre a massa micelial seca, a germinação de conídios e a conidiogênese de *Cladosporium* spp. também foram investigados utilizando um hemocitômetro. Os valores de CIM e CFM do citronelol variaram de 256 a 512 µg/mL e de 256 a 2048 µg/mL, respectivamente. CIM e CFM de geraniol variaram de forma semelhante. A germinação de conídios, massa micelial seca e conidiogênese de *Cladosporium* spp. foram inibidas pelas drogas-teste 1/2CIM, CIM e 2xCIM ($p<0,05$). Esses eventos celulares são essenciais para a infecção e desenvolvimento fúngico em alimentos. A ação de citronelol e geraniol contra *Cladosporium* spp. sugere que podem servir como agentes eficazes para controlar a contaminação fúngica e o seu crescimento em alimentos.

Palavras-chave. monoterpenos, contaminação, produtos naturais, segurança alimentar, *Cladosporium*.

INTRODUCTION

Food acts as a vehicle for many microorganisms; causatives of food-borne diseases. Among these, fungal contaminants are worth mentioning since they can cause food spoilage, allergic reactions, poisoning, and infections in humans and animals[1].

Cladosporium spp., dematiaceous fungi, are contaminants found as saprophytes in soil and decaying materials, particularly in temperate regions, such as the semi-arid region of Brazil. The species most often isolated are *Cladosporium elatum, C. herbarum, C. spherospermum, C. cladosporioides, C. carrionii,* and *C. oxysporum*[2-4]. *Cladosporium* species are producers of volatile organic compounds associated with the odors of decaying food. Fungal growth on food modifies its maturation, causes discoloration, unpleasant odor, chemical and nutritional changes, and loss of quality. Fungi also cause losses to producers, to industry, and to consumers.[5,6] Further, the genus *Cladosporium* produces several secondary metabolites; mycotoxins such as cladosporin and emodin, which are both heat resistant and cause serious problems to internal human organs[5].

Considering the importance of fungal contamination, procedures to control fungal growth are fundamental to prevent foodborne illness and food quality losses. One of the principal methods is application of chemical sanitizers such as chlorine or quaternary ammonium products. However, to provide microbiologically safe foods, the demand for natural alternatives to replace chemically synthesized antimicrobials has been increasing[7].

In this context many studies have been conducted with natural substances, like the terpenes, to investigate possible applications in microbiological food control. Terpene compounds, widely distributed in plants, are hydrogenated cyclic or aliphatic carbon chains endowed with activities against pathogenic fungi and contaminants[8]. Within the group, those oxygenated due to the action of specific enzyme systems such as citronellol and geraniol are called monoterpenoids (C_{10}). Monoterpenoids are a group of molecules with antimicrobial activity[9].

This study presents the antifungal potential of citronellol and geraniol on strains of the genus *Cladosporium*; focusing on inhibitory effects against important stages of fungal development such as spore germination and production, and mycelial formation. These monoterpenoids have demonstrated various biological activities; researchers have shown that geraniol has outstanding neuroprotective[10], insecticidal[11] and antineoplastic activities[12]. Currently, there are reports in the literature confirming the antifungal potential of geraniol against strains of *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp.[13]. Meanwhile, it is reported that citronellol has: anti-hyperalgesic[14], antimicrobial[13] and repellant activity[15].

MATERIALS AND METHODS

Fungal strains

For the antifungal activity tests, the strains: *C. carrioni* (LM 227, URM 5109), *C. cladosporioides* (URM 5737, URM 6246), and *C. oxysporum* (URM 5234, URM 5412), were taken from the culture collections of the (Federal University of Paraíba) Mycology Laboratory, and the (Federal University of Pernambuco) Department of Mycology. The fungi were grown in potato dextrose agar at 28 °C for 7 days. Fresh cultures were overlaid with sterile saline (0.9% NaCl), and suspensions formed by gentle agitation. The resulting mixture of conidia and hyphal fragments were transferred to sterile test tubes. After 15 seconds of stirring, each inoculum was allowed to stand for 3 minutes and the supernatant was collected in sterile test tubes. After stirring, the conidia were counted using a hemocytometer and adjusted to an inoculum of approximately 10^6 conidia/mL[16,17].

Test compounds

Citronellol and geraniol were purchased from Sigma-Aldrich® (Brazil). Emulsions were freshly prepared for the tests by first dissolving them in dimethylsulfoxide (DMSO), with sterilized distilled water to obtain a concentration of 1024 µg/mL. From this concentration, dilutions were performed to achieve a concentration of 1 µg/mL using RPMI 1640 medium.

Determination of minimum inhibitory concentration (MIC)

Determining the MIC for each test-drug was carried out by microdilution technique using 96 well flat bottom micro-titer plates as adapted from document M38-A of the CLSI[18]. To each row of the plate was added 100 μL of the diluted test drugs in RPMI 1640. To each well of the plate was added 100 μL of a previously prepared inoculum diluted in RPMI 1640 to a ratio of 1:50. A fungal control was performed by replacing the test drug with sterile saline (growth control). A sterility control and DMSO were also performed. The plates were sealed and incubated at 28 °C for 5 days. The MIC values were determined by visual analysis of growth inhibition in each well, as compared to controlled growth. The MIC was the lowest concentration of drugs capable of inhibiting observed fungal growth in the wells by 100%. The experiment was performed in triplicate and the MIC values were expressed as a geometric mean.

Determination of minimum fungicide concentration (MFC)

A 10 μL aliquot from each well having no fungal growth was sown on a plate with Sabouraud dextrose agar. The plates were incubated at 28 °C for 5 days. The MFC was considered to be the lowest concentration sown, where the growth was less than 3 colony-forming units. The experiment was performed in triplicate and the MFC values were expressed as a geometric mean[19,20].

Effects on conidia germination

In sterile test tubes, 500 μL of the RPMI 1640 plus the test-drugs (1/2MIC, MIC, 2xMIC) were homogeneously mixed with 500 μL inoculates of *C. carrioni* LM 227, *C. cladosporioides* URM 5737, and *C. oxysporum* URM 5234. The tubes were incubated at 28 °C for 48 hours. The quantities of germinated and non-germinated conidia were determined using a hemocytometer. The percentages of germinated conidia were calculated for each experimental group. A control with no drug was used. The whole experiment was performed in triplicate[21,22].

Effects on dry mycelial weight

The analysis of test-drug interference on mycelial growth was performed by determining the dry mycelial masses for *C. carrioni* LM 227, *C. cladosporioides* URM 5737, and *C. oxysporum* URM 5234[22,23]. To a sterile test tube were added 2.5 mL of RMPI 1640, previously completed with the test-drug solutions (1/2MIC, MIC, 2xMIC), and then 0.5 mL of inoculum to each tube. The controls (no drugs) were performed in a similar manner. The system was incubated at 28 °C for 5 days to determine the dry mycelial mass. For this, the cultures were sterile filtered using filter paper (porosity: 11 μm), and washed with distilled sterile water. The mycelium retained in the filter paper was subjected to drying in an oven at 60 °C for 10 minutes. Upon completion, the filter paper containing the dry mycelium was weighed and the dry mycelial mass percentage was calculated considering the experimental control as 100% mycelial production. The experiment was performed in triplicate.

Effects on fungal conidiogenesis

C. carrioni LM 227, *C. cladosporioides* URM 5737, and *C. oxysporum* URM 5234 conidia production was analyzed (after cultivation) on Sabouraud dextrose agar in the absence and presence of test-drugs (at 1/2MIC, MIC, 2xMIC) according to Tzortzakis and Economakis[24]. Into sterile test tubes, 10 mL of Sabouraud dextrose agar was poured and set in a water bath at 35 °C. The test drugs were then added. The controls (no drugs) were performed in a similar manner. On the surface of the medium was placed a 2 mm portion of fungal mycelium, newly grown in potato dextrose agar, and the whole system incubated at 28 °C for 5 days. Afterwards, the conidia were collected by adding 5 mL of sterile saline solution to the surface of the fungal colonies. The suspension was then collected and centrifuged at 4500*g* for 5 minutes, the supernatant was then discarded and the pellet washed once with sterile saline. The inoculum was analyzed in a hemocytometer to count the number of conidia in each group tested. Assays were performed in triplicate.

Statistical analysis

The results were expressed as mean ± SEM. The data were compared statistically using the unpaired *t*-test. A $p<0.05$ was considered significant.

RESULTS

Initially, tests were performed to determine the MFC and MIC of citronellol and geraniol against the *Cladosporium* spp. strains. As can be seen in **Table**, the test-drugs inhibited the growth of the tested strains starting from the 256 µg/mL concentration. Importantly, the species *C. carrioni* was the most sensitive, having lower MIC values for both drugs. The control tests, confirmed the following: (1) fungal growth in the absence of the drugs; revealing the viability of the fungal inoculum (growth control), (2) the absence of fungal growth in the culture medium without inoculum (sterility control), (3) fungal growth when DMSO was used in the same concentrations as the drug emulsifier. The minimum fungicidal concentration (MFC) of citronellol was found in concentrations above the respective MIC values when tested against the strains of *C. cladosporioides* and *C. oxysporum*. For the *C. carrioni* strains, the MIC of citronellol was identical to the MFC. Geraniol showed similar results.

Our results showed that at all concentrations tested, citronellol and geraniol significantly inhibited ($p<0.05$) the mycelial growth of *C. cladosporioides* URM 5737, and *C. oxysporum* URM 5234 as compared to the controls. Regarding the strain *C. carrioni* LM 227, geraniol only showed inhibition ($p<0.05$) at the MIC and 2xMIC concentrations (**Figure 1a**).

Table. Minimum inhibitory concentration (MIC) and minimum fungicide concentration (MFC) of citronellol and geraniol against *Cladosporium* spp. strains

Fungi	Citronellol (µg/mL)		Geraniol (µg/mL)	
	MIC	MFC	MIC	MFC
C. carrioni LM 227	256	256	512	512
C. carrioni URM 5109	256	256	256	512
C. cladosporioides URM 5737	512	1024	512	512
C. cladosporioides URM 6246	256	512	512	512
C. oxysporum URM 5234	512	2048	512	2048
C. oxysporum URM 5412	512	1024	512	1024

All concentrations of the test-drugs inhibited the conidia germination processes of *C. cladosporioides* URM 5737, *C. carrioni* LM 227 and *C. oxysporum* URM 5234 when compared to the test controls (absence of the drug) ($p<0.05$). The results also showed that all groups present inhibition of conidial production ($p<0.05$), except geraniol (at 1/2MIC), which did not significantly inhibit conidiogenesis for *C. oxysporum* URM 5234 as compared to the control (**Figure 3c**).
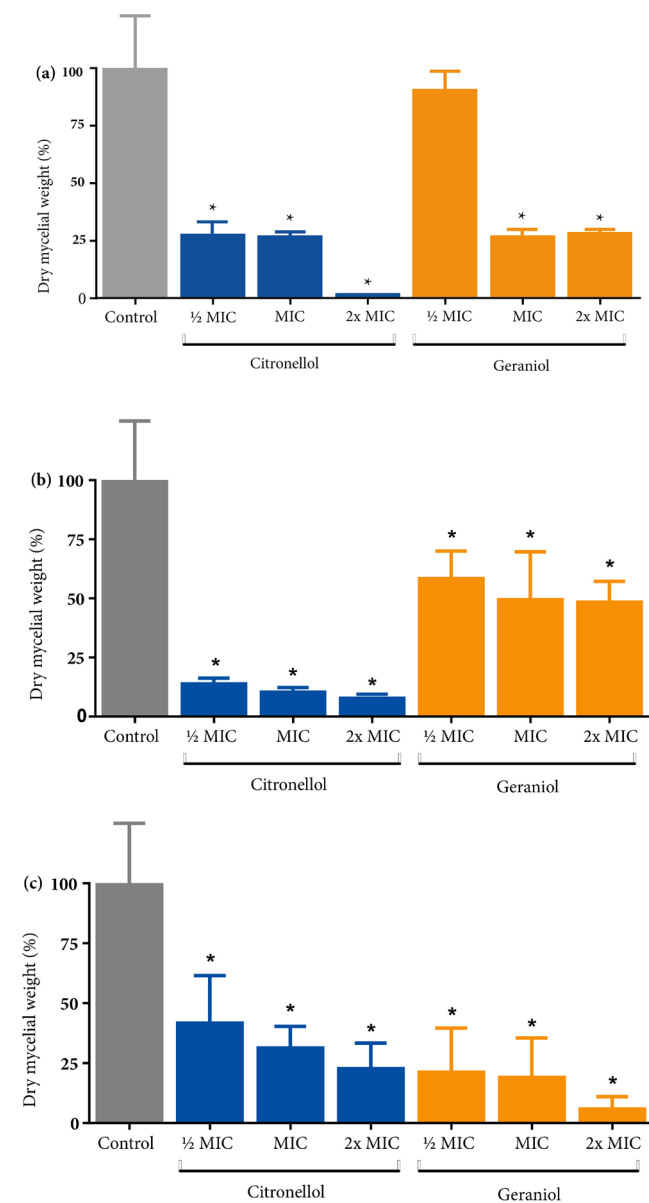

Figure 1. Percentage of dry mycelial weight produced by *Cladosporium carrioni* LM 227 (a), *C. cladosporioides* URM 5737 (b) and *C. oxysporum* URM 5234 (c) in the absence (control) and presence of citronellol and geraniol. Control produced 100% of dry mycelial weight. *$p<0.05$ compared to control
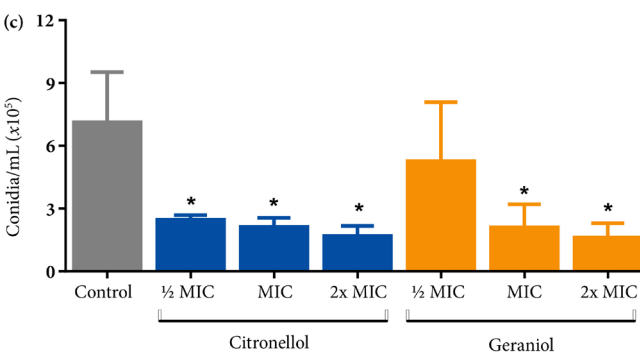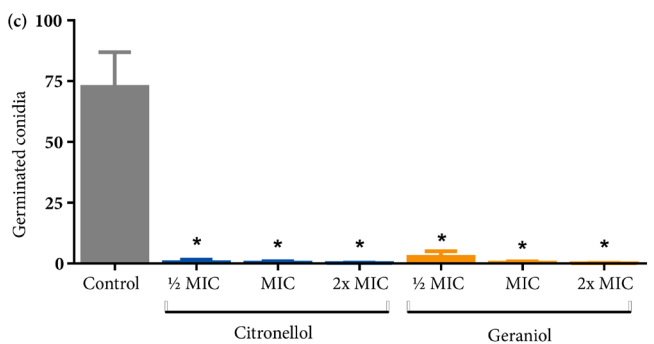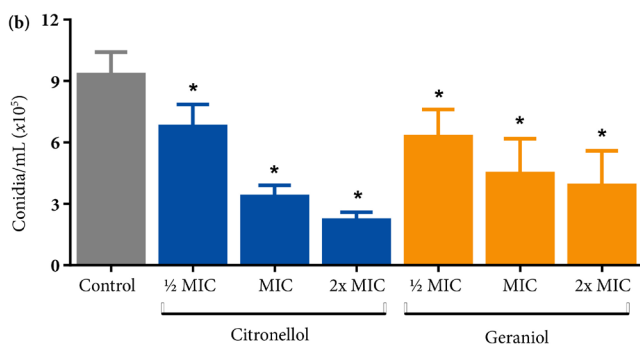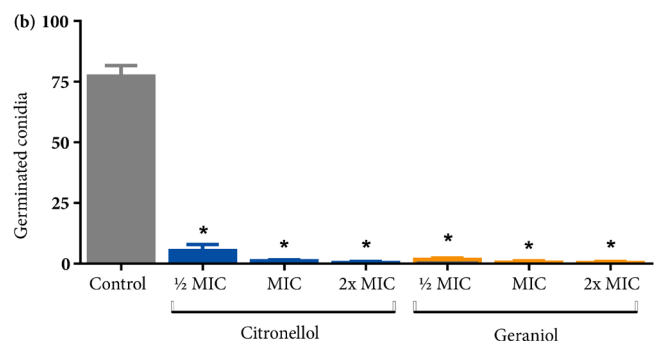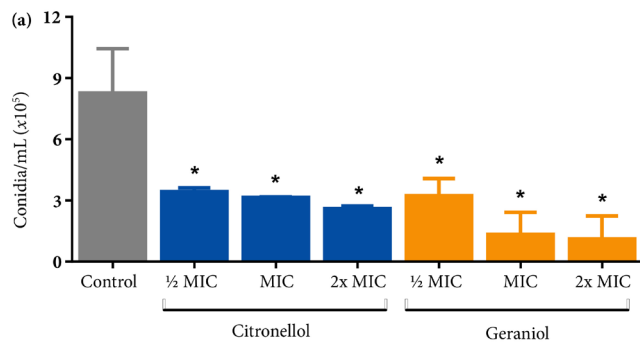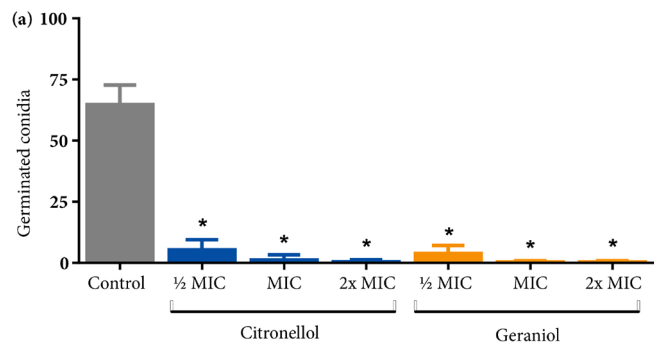
Figure 2. Percentage of germinated conidia of *Cladosporium carrioni* LM 227 (a), *Cladosporium cladosporioides* URM 5737 (b) and *Cladosporium oxysporum* URM 5234 (c) in the absence (control) and presence of citronellol and geraniol. *p<0.05 compared to control

Figure 3. Number of conidia/mL produced by *Cladosporium carrioni* LM 227 (a), *C. cladosporioides* URM 5737 (b) and *C. oxysporum* URM 5234 (c) in the absence (control) and presence of citronellol and geraniol. *p<0.05 compared to control

DISCUSSION

According to Aoudou et al[13], when antimicrobial agents are tested, it is common to obtain fungicidal values which are higher than their MIC values, validating our results. In our study, citronellol and geraniol showed outstanding antifungal activity against all of the strains tested. However, according to criteria proposed by Sartoratto et al[25], both compounds presented strong antifungal activity.

This unique character makes the results of this study highly relevant. However, the antifungal potential of geraniol and citronellol has been reported in other studies such as Shin and Lim[26], which demonstrated such antifungal activity against the dermatophyte species: *Trichophyton erinacei, T. mentagrophytes, T. rubrum, T. tonsurans, T. schoenleinii* and *T. soudanense*; also using microdilution technique. Pereira et al[27] reported evidence that both drugs interfere with *T. rubrum* growth by inhibiting ergosterol biosynthesis. Aoudou et al[13] also examined antifungal activity against contamination with *Fusarium, Aspergillus*, and *Penicillium* species. In addition to the antifungal activity of the isolated components, their essential oils (high in citronellol and geraniol) also have proven antifungal activity against many species. The essential oil of *Cymbopogon winterianus* Jowitt ex. Bor has large amounts of geraniol and citronellol, and is therefore recognized as having antifungal activity[22,28].

To study the interference of the test-drugs at different stages of fungal development such as fungal mycelial growth, conidia germination, and conidiogenesis, we chose the strains *C. cladosporioides* URM 5737, *C. carrioni* LM 227 and 5234 *C. oxysporum* URM; because of their higher growth rates. Our results were promising, since the drugs inhibited cellular processes.

The inhibitory effects of natural drugs on mycelium, conidia production, and germination of conidia for *Trichophyton rubrum*[27], *Rhizopus oryzae*[17], *Sclerotium cepivorum*[29] have been previously reported. There is evidence in the literature that due to the lipophilic profile of terpenes, their antifungal mode of action involves disruption of the plasma membrane lipid bilayer. A subsequent release of intracellular components, and disruption of the activity of membrane enzymes, interferes with energy dependent processes such as: solute transport, metabolism regulation, ergosterol synthesis, cell wall formation and morphogenesis[8,27].

The air itself can be an important distributor of fungal conidia. Once produced and then dispersed, conidia start the process of germination in the presence of nutrients such as amino acids and sugars present in food. Morphological changes in the single asexual fungal cell provide longitudinal growth in hyphae. Hyphae penetrate the inner layers of food, increasing the extent of injury, and cause deterioration; with consequent losses[30-32].

For years, researchers have been seeking an alternative to applying chlorine-based compounds; or new ways to control fungi contaminants. Currently, the safety of such chemical preservatives is being debated, since many of these products have both carcinogenic and teratogenic properties, as well as residual toxicity[33,34].

Belsito et al[35] published a review showing that acute oral toxicities of non-cyclic terpene alcohols, including geraniol and citronellol, present low LD_{50} values in rats. These safety data are relevant for risk assessments of monoterpene alcohol use in the food industry. The use of natural antimicrobial agents for food preservation is a globally recognized control measure; to be used either alone or in combination with other preservation technologies. Although many reports highlight terpenes and essential oils as potential flavoring agents, they also represent a source of natural drugs with antifungal potential. Yet, possible application as food preservatives requires specific knowledge of their properties, such as those presented in this study. Further, their organoleptic effects on the food matrix must also be analyzed[8].

CONCLUSION

The formation of fungal conidia and their subsequent germination drive mycelium formation, and therefore, the appearance of fungal food infections. Our results are relevant because they demonstrate that both citronellol and geraniol effectively interfere in these growth processes. In conclusion, we present two natural drugs with anti-*Cladosporium* potential; citronellol and geraniol, which may well be useful in the food industry. However, even with roof of their antifungal potential, further

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studies are necessary so hat the drugs may be applied appropriately as preservatives in food.

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